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**ORIGINAL ARTICLE****EFFECT OF GREEN TEA EXTRACT SUPPLEMENTATION IN THE SEMEN EXTENDER ON POST-THAW SPERM QUALITY OF SIMMENTAL BULLS**

Suherni Susilowati, DVM, MKes<sup>1</sup>, Trilas Sardjito, DVM, MSi<sup>1</sup>, Oky Setyo Widodo, DVM, MSi<sup>2</sup>, Rochmah Kurnijasanti, DVM, MSi<sup>3</sup>, Wurlina Wurlina, DVM, MSi<sup>1</sup>, Erma Safitri, DVM, MSi<sup>1</sup> and Imam Mustofa, DVM, MKes<sup>1\*</sup>

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**ABSTRACT**

The aim of this study was to determine the effects of green tea extract as antioxidant in the extender of frozen semen on the viability, motility, integrity of plasma membrane, percentage of necrosis and apoptosis of spermatozoa. Ejaculates collected from four Simmental bulls were evaluated and placed in 37°C water bath before the cryopreservation process was started. The semen samples were diluted in milk-egg yolk supplemented with green tea extract of 0 (P0) as control, 0.05 (P1), 0.1 (P2), and 0.15 (P3) mg/100 ml extender, respectively. Diluted semen packed in 0.25 ml straws were cryopreserved and stored in liquid nitrogen for four weeks. Cryopreserved semen packed in straws were thawed individually in 37°C water bath for 30 sec then evaluated microscopically. The results showed that P2 had the highest post-thaw sperm viability, motility, and integrity of plasma membrane compared to the other groups ( $P < 0.05$ ). In addition, the percentage of necrosis and apoptosis of sperm were lowest in P2 ( $P < 0.05$ ) compared to the other groups. The results of the study suggest that semen samples diluted in milk-egg yolk supplemented with green tea extract at the rate of 0.1 mg/100 ml extender had the highest semen quality. Thus, green tea extract can potentially act as antioxidant to maintain the quality of post-thaw bull spermatozoa.

**Key words:** apoptosis, bull, green tea extract, necrosis, post-thaw, sperm quality

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**INTRODUCTION**

Genetic improvement is a key in any modern cattle breeding system. One way to achieve genetic improvement is to use cryopreserved semen of superior commercial sires (Biscarini *et al.*, 2015). The effects of cryopreservation on sperm function and fertility have been widely studied. Techniques for the successful cryopreservation of spermatozoa have progressed and are now standardized (Barik *et al.*, 2016). Cryopreserved semen may be used

for a long time. However, a decrease in sperm quality after the cryopreservation process may be observed. About 50% of spermatozoa will die during the cryopreservation process and spermatozoa that survive generally have low fertility (Lessard *et al.*, 2000). During the process of cryopreservation and thawing, the spermatozoa pass through a variety of temperature changes and extreme osmolarity that could trigger the production of reactive oxygen species (ROS) (Moore *et al.*, 2005; Nebel, 2007). Thus, cryopreservation of spermatozoa also has detrimental effects on sperm quality

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after thawing (Martinez-Alborcia *et al.*, 2012). During cryopreservation, semen is exposed to cold shock and atmospheric oxygen, which increases the level of lipid peroxidation (Agarwal *et al.*, 2014). Production of reactive oxygen species (ROS) during cryopreservation has been associated with deterioration of sperm motility, viability, plasma membrane integrity (Ansari *et al.*, 2011) and promote DNA fragmentation of spermatozoa (Baumber *et al.*, 2003). Experiments have shown that the oxidative stress produced due to high reactive oxygen species (ROS) is associated with low quality of seminal plasma (Bailey *et al.*, 2000).

Green tea extract is one of the plant-derived antioxidants. Several studies have documented that herb antioxidants in semen extender were associated with the improvement of spermatozoa quality (Khan *et al.*, 2011). Based on this, it is necessary to determine the effect of green tea extract supplementation as antioxidants in the cryopreservation of bull spermatozoa.

## MATERIAL AND METHODS

This research was conducted at Singosari National Artificial Insemination Center, Malang, East Java, Indonesia. Four 3-5 year-old, 400-900 kg fertile Simmental bulls were used in this study. Semen was collected from the bulls twice a week by using artificial vagina. Fresh semen was assessed macroscopically (volume, odor, pH, consistence and color) and microscopically (motility, viability and concentration of the sperm). If spermatozoa motility and viability are more than 70%, then the semen qualifies for the study. The ejaculate was cryopreserved according to The Indonesian National Standard number SNI: 4869-1:2017 (production and analysis of frozen semen) of The National Standardization Agency of Indonesia.

### Processing of semen

Milk-egg yolk extender containing penicillin (1000 IU/ml), streptomycin (1 mg/ml), 7% glycerol and distilled water were added to reach 100 ml. The ejaculate from each bull was divided into four equal extender groups, *i.e.* control group (P0) and three treatment groups

supplemented with 0.05 (P1), 0.1 (P2) and 0.15 (P3) mg/100 ml extender, respectively. Fresh semen was diluted in extender to obtained  $30 \times 10^6$  spermatozoa/ml.

Each diluted semen from each group was equilibrated at 5°C for 2 h before being packaged in 0.25 ml semen straws. The straws were placed on steel racks (Cooltop, Minitube) and held in liquid nitrogen vapor at -140°C for 10 min. Thereafter, the straws were immediately immersed in liquid nitrogen (-196°C) and stored for 4 weeks until further assessment. Post-thaw semen analysis was conducted on two samples of straws from each treatment. Cryopreserved semen was thawed at 37°C for 30 sec to determine the semen quality parameters with six replications.

### Green tea extract preparation

Green tea leaves were dried, milled with a grinding machine, and then soaked with 96% ethanol and allowed to stand for 3 days, and covered with aluminum foil to prevent evaporation. Furthermore, the green tea solution was squeezed by using filter paper, and then the filtrate was evaporated at 50°C and centrifuged at 45 rpm to separate the solvent from the extract of green tea leaves to obtain thickened extract. The extract was evaporated in an acid chamber until solid extract was formed and then freeze dried.

### Sperm motility

Progressive motility was assessed using a phase contrast microscope (200× magnification) at 37°C. A wet mount was made by using a 10 µl drop of semen placed directly on a microscope slide and covered with cover glass (Asia *et al.*, 2017). Sperm motility was counted on three microscopic fields for each semen sample and then averaged.

### Sperm viability

Eosin negrosin staining was used to evaluate the viability (live/dead in %) of semen. One drop of semen was placed on an object glass slide and mixed with one drop of eosin-negrosin solution. The mixture was smeared on the glass slide and allowed to dry. One hundred spermatozoa were evaluated in at least five different fields in each smear under a light microscope. Observed and recorded

spermatozoa with unstained heads were evaluated as live, while those with stained or partially stained heads were evaluated as dead spermatozoa (Asia *et al.*, 2017).

### **Sperm plasma membrane integrity**

Hypo-osmotic swelling (HOS) test was used to evaluate plasma membrane integrity (Gohar *et al.*, 2014). One ml of hypo-osmotic solution (7.35 g of sodium citrate, 13.52 g of fructose dissolved in 1000 ml of aquadest) was added to 0.1 ml of spermatozoa, then incubated at room temperature for 30 min. Following incubation, 15 µl of the sample was placed on an object glass slide, covered with a cover glass and observed using a 400× magnification microscope. Spermatozoa with intact plasma membrane are characterized by a circular spermatozoa tail while damaged spermatozoa membrane is characterized by a straight tail (Ramu and Jeyendran, 2013).

### **Sperm necrosis**

One drop of semen was smeared on the object glass, fixed in solution of glacial acetic acid and absolute methanol (1:3) for 15 min, and finally stained with Hematoxylin Eosin. Necrotic spermatozoa are characterized by pycnosis, karyorrhexis, and karyolysis of nuclei (Mulyati *et al.*, 2016). Examination was conducted on five different fields at 400× magnification per 100 spermatozoa under phase-contrast microscope (Olympus BX51 TF, Japan).

### **Sperm apoptosis**

Acridin Orange Staining was used to determine apoptosis of spermatozoa. One drop of semen was smeared on the object glass, and dried at room temperature. The smear preparations were fixed in solution of glacial acetic acid and absolute methanol (1:3) for 15 min, dried again at room temperature, dipped in acridin orange, and then covered with a cover glass. Observation was conducted under a fluorescence microscope with 100× magnification per 100 spermatozoa. Apoptotic spermatozoa were yellow-red, while the live spermatozoa were greenish in color (Ortega-Ferrusola *et al.*, 2017).

### **Statistical analysis**

All data were expressed as mean value±SD. Analysis of variance (ANOVA) at 5% level of significance was used to determine if there are significant differences among treatment means. Student's t-test was used to determine differences between mean values.

## **RESULTS AND DISCUSSION**

In this study, macroscopic and microscopic evaluation of fresh Simmental bull semen was conducted if they can qualify for processing into frozen semen. The macroscopic evaluation consisted of examination of volume, color, odor, consistency and degree of acidity (pH), while microscopic evaluation was examination of mass movement, individual movement, concentration and viability of spermatozoa (Table 1). The semen volume was 7.26 ml, consistency was medium to thick, color was milky white to yellowish and odor was specific smell of bull semen. The semen had a pH of 6-7 and concentration of 1.04 billion/ml. The spermatozoa had a motility of 85.83%, viability of 95.65% and abnormality of 6.69%.

Based on macroscopic and microscopic evaluation, the fresh Simmental bull semen qualified for cryopreservation. Sperm motility is different from its viability because a living sperm is not necessarily mobile whereas a motile spermatozoa is definitely alive (Mulyati *et al.*, 2016). The motility of spermatozoa is one of the criteria of semen quality that correlates with the ability of spermatozoa to fertilize the ovum. The motility of spermatozoa is due to the energy (Adenosine Triphosphate) produced by the mitochondria and is also due to the dynein motor (cytoskeleton) for flagella where motion is regulated by  $Ca^{2+}$  and cyclic Adenosine Monophosphate (cAMP) (Pereira *et al.*, 2017).

Spermatozoa absolutely require an intact plasma membrane integrity for survival. Plasma membrane is the entrance of substances from the exterior to the interior of the cell, or vice versa. If the plasma membrane of spermatozoa is damaged, its metabolism will be disrupted, resulting in decreased motility and ability of spermatozoa to fertilize the egg (Garner and Hafez, 2013).

Functional assay of spermatozoa in pre-



Table 1. Characteristics of Simmental bull semen used in the study.

Parameter	Mean $\pm$ SD
Volume (ml)	7.26 $\pm$ 1.722
Consistency	Medium to thick
Color	Milky white to yellowish
Odor	Specific smell of bull semen
pH	6-7
Mass Movement	+++
Motility (%)/speed	85.83 $\pm$ 2.896/3
Viability (%)	95.65 $\pm$ 2.363
Abnormality (%)	6.69 $\pm$ 1.867
Concentration (millions/ml)	1040 $\pm$ 226.551

cryopreserved and post-thawed semen showed that supplementation of 0.1 mg/100 ml green tea extract in milk-egg yolk semen extender had the highest viability, motility (Table 2) and plasma membrane integrity, and the lowest necrosis and apoptosis of spermatozoa compared to other groups (Table 3) ( $P < 0.05$ ).

Cryopreservation process leads to damage mainly caused by the cold shock, intracellular ice crystal formation, oxidative stress, and reorganization of lipid and protein of the plasma membrane (Bailey *et al.*, 2000). Cryopreservation can damage the spermatozoa cell compartment such as membranes (plasma and acrosome), mitochondria and even chromatin (Castro *et al.*, 2016). This is

because during the process of cryopreservation and thawing of spermatozoa, it goes through a variety of temperature changes and extreme osmolarity which trigger the production of reactive oxygen species (ROS) (Nebel, 2007; Moore *et al.*, 2005). Stress on the plasma membrane during cryopreservation of spermatozoa leads to asymmetric changes in the phospholipid bilayer membrane. The plasma membrane of mammalian spermatozoa is rich in polyunsaturated fatty acids that are easily damaged by Reactive Oxygen Species (ROS) in a reaction called lipid peroxidation (Agarwal *et al.*, 2003).

Lipid peroxidation during the semen cryopreservation process will damage the

Table 2. Viability and motility of pre-cryopreserved and post-thawed spermatozoa of Simmental bull semen diluted in milk-egg yolk extender.

	PO	P1	P2	P3
Viability				
Before cryopreservation	64.25 $\pm$ 2.057 <sup>a</sup>	68.20 $\pm$ 1.706 <sup>b</sup>	77.50 $\pm$ 2.355 <sup>c</sup>	68.55 $\pm$ 2.204 <sup>b</sup>
Post-thawed	58.15 $\pm$ 1.259 <sup>a</sup>	58.35 $\pm$ 1.151 <sup>a</sup>	68.25 $\pm$ 1.109 <sup>b</sup>	59.15 $\pm$ 1.206 <sup>a</sup>
Motility				
Before cryopreservation	60.25 $\pm$ 2.353 <sup>a</sup>	65.20 $\pm$ 1.701 <sup>b</sup>	75.50 $\pm$ 2.503 <sup>c</sup>	66.55 $\pm$ 2.402 <sup>b</sup>
Post-thawed	55.45 $\pm$ 1.256 <sup>a</sup>	58.35 $\pm$ 1.103 <sup>b</sup>	64.35 $\pm$ 1.206 <sup>c</sup>	58.15 $\pm$ 1.301 <sup>b</sup>

Values with different superscripts in the same row are significantly different ( $P < 0.05$ ).

P0: control, P1, P2, and P3 supplemented with green tea extract at 0.05, 0.10, and 0.15 mg/100 ml extender, respectively.



Table 3. Intact plasma membrane integrity, necrosis and apoptosis of pre-cryopreserved and post-thawed spermatozoa of Simmental bull semen diluted in milk-egg yolk extender.

	PO	P1	P2	P3
Intact plasma membrane integrity				
Before cryopreservation	56.25±2.057 <sup>a</sup>	62.20±1.708 <sup>b</sup>	72.25±2.152 <sup>c</sup>	63.35±2.204 <sup>b</sup>
Post-thawed	46.15±1.252 <sup>a</sup>	50.35±1.155 <sup>c</sup>	62.25±1.158 <sup>d</sup>	48.15±1.206 <sup>b</sup>
Necrosis				
Before cryopreservation	5.25±2.058 <sup>c</sup>	3.20±1.709 <sup>b</sup>	2.25±0.153 <sup>a</sup>	4.35±2.202 <sup>b</sup>
Post-thawed	7.15±1.154 <sup>c</sup>	6.35±1.252 <sup>b</sup>	3.05±1.153 <sup>a</sup>	5.15±1.207 <sup>b</sup>
Apoptosis				
Before cryopreservation	8.15±2.052 <sup>c</sup>	4.20±1.701 <sup>b</sup>	3.25±0.153 <sup>a</sup>	5.25±2.207 <sup>b</sup>
Post-thawed	9.15±1.251 <sup>c</sup>	7.35±1.303 <sup>b</sup>	4.05±1.158 <sup>a</sup>	7.05±1.257 <sup>b</sup>

Values with different superscripts in the same row are significantly different (P<0.05).

P0: control, P1, P2, and P3 supplemented with green tea extract at 0.05, 0.10, and 0.15 mg/100 ml extender, respectively.

spermatozoa, because the plasma membrane of spermatozoa is composed of phospholipid. It contains unsaturated fatty acids which is particularly susceptible to free radicals that will stimulate the autocatalytic reactions and cause damage at the double bonds (Catalá, 2013). Lipid peroxidation may alter the structure of plasma membrane, the region of spermatozoa acrosome and cause the loss of motility. Prolonged lipid peroxidation may damage the structure of the lipid matrix which will cause an unstable cell plasma membrane (Itri *et al.*, 2014).

ROS plays a role in the regulation of spermatozoa function physiologically. ROS affect post ejaculated spermatozoa motility initiation through cAMP synthesis and protein phosphorylation (Baumber *et al.*, 2003). Superoxide anion at physiologic concentrations is required for initiation of hyper-activation, capacitation and acrosomic reactions during fertilization (Ogbuewu *et al.*, 2010 ; Thompson *et al.*, 2014). Other studies have suggested that low concentrations of free radicals in human semen increase the ability of spermatozoa to bind to the zona pelucida (Kumar and Das, 2005). The concentration of antioxidant and storage periods have significant effects on motility, viability, acrosome integrity and membrane integrity (Itri *et al.*, 2014). This

means that anti-oxidants prevent the increase of free radicals and improve the quality of semen (Ahmadi *et al.*, 2016).

There are biological antioxidants such as GSH, glutathione peroxidase, catalase, and superoxide dismutase (SOD) in the semen that have important roles as suppressors of free radicals (Petruska *et al.*, 2014). Endogenous antioxidant capacity of spermatozoa may be disrupted during cryopreservation and thawing process. If the balance between Reactive Oxygen Species (ROS) and antioxidants is disturbed, oxidative stress may result in negative effects on DNA integrity (Baumber *et al.*, 2003), inhibition of oxidative metabolism (Makker *et al.*, 2009) and decreased motility and viability of spermatozoa (Agarwal *et al.*, 2014).

Recently, plant-derived antioxidants have been getting major focus due to their lower cytotoxicity and are considered to be better than synthetic antioxidants (Ibrahim *et al.*, 2014). Green tea has valuable effects on health due to the abundant amount of polyphenols that are the major water soluble components of green tea infusions (Lee *et al.*, 2014). In addition, green tea polyphenol (GTP) is potent in decreasing ROS (Roy *et al.*, 2003). One of the products from tea leaves (*Camellia sinensis*) processing is green tea

which has strong antioxidant activity. Green tea extract contains high concentration of liquid polyphenol (Senanayake, 2013). The main polyphenols in green tea are epicatechin (EC), epigallocatechin (EGC), epicatechin galat (ECG) and epigallocatechin galat (EGCG) (Du *et al.*, 2012).

The results of this study showed that supplementation of green tea extract in milk-egg yolk semen extender could maintain motility, viability, integrity of the plasma membrane and decrease the necrosis and apoptosis of Simmental bull spermatozoa. The best dose was 0.1 mg/100 ml extender. The beneficial effects of green tea seems to be not dose-dependent. It maybe that green tea extract more than 0.1 mg / 100 ml extender was toxic for the sperm.

Green tea extract contains polyphenols which are antioxidants that inhibit the peroxidation reaction where free radicals are not formed. Moreover, the presence of active substances, catechin in green tea extract can suppress ROS. The catechin compound could overcome the presence of superoxide, hydroxyl radicals, peroxy radicals and free radicals. Catechin stopped the attacks of oxidative radicals on the cell membrane that resulted to the prevention of cell death. Cell membranes remain undamaged and can function properly to regulate inflow and outflow of substrate needed in the metabolic processes. The hydroxyl group of catechin compounds acts as an antioxidant capable of reducing the production of ROS (Armoskaite *et al.*, 2011).

The supplementation of green tea extract in milk-egg yolk semen extender could maintain motility, viability and plasma membrane integrity and decrease necrosis and apoptosis of post-thawed Simmental bull spermatozoa. The best dose of green tea extract in milk-egg yolk semen extender observed in this study is 0.1mg/100 ml of semen extender.

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